

## Existence of Three Mechanisms for Blocking Polyspermy in Oocytes of the Mussel *Mytilus edulis*

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**Abstract.** We found the existence of a three-step mechanism to block polyspermy in the oocyte of the mussel *Mytilus edulis*. When the oocytes were inseminated within 30 min after spawning, they underwent monospermic fertilization over a wide range of sperm-oocyte ratios up to  $5 \times 10^3$ . A transient depolarization of the oocyte plasma membrane (fertilization potential) was observed immediately after insemination. Low-sodium seawater induced polyspermy and decreased the amplitude of the fertilization potential, suggesting the existence of a fast block to polyspermy that is dependent on depolarization of the plasma membrane. When the fertilized oocytes were inseminated again at a sperm-oocyte ratio that is great enough to give a high rate of polyspermy in initial insemination, many sperm could not undergo the acrosomal reaction and thus could not penetrate fertilized oocytes. The remaining sperm underwent an acrosomal reaction and the acrosomal process protruded through the vitelline coat, but it did not fuse with the oocyte plasma membrane. These findings suggest the existence of two strategies constituting a late polyspermy block: suppression of acrosomal reaction and block of contact or fusion between the plasma membranes of sperm and oocyte.

### Introduction

The success of fertilization and the subsequent development of the zygote require the fusion of a single male pronucleus with a female pronucleus. Penetration of the

spermatozoon into an oocyte is controlled through several mechanisms (Jaffe and Gould, 1985). Rothschild and Swann (1952) first suggested the existence of a fast polyspermy block at the plasma membrane of the oocyte in the sea urchin. Jaffe (1976) demonstrated that the block was mediated electrically. The fast electrical polyspermy block has been further observed in many animal species, such as starfish (Miyazaki and Hirai, 1979), the echiuroid *Urechis* (Gould-Somero *et al.*, 1979), amphibians (Cross and Elinson, 1980; Grey *et al.*, 1982; Iwao, 1989), the nemertean *Cerebratulus* (Kline *et al.*, 1985), crab (Goudeau and Goudeau, 1989a), lamprey (Kobayashi *et al.*, 1994), and ascidians (Goudeau *et al.*, 1994), as well as in algal protists, among the furoid seaweeds (Brawley, 1991).

After fusion between the plasma membranes of the gametes, the extracellular coat of the oocyte is altered and forms a fertilization envelope to prevent sperm penetration (zona reaction in mammals). A late polyspermy block mechanism may also operate at the level of the oocyte plasma membrane in *Urechis*. Paul and Gould-Somero (1976) found that the acrosomal process of supernumerary sperm penetrated through the vitelline coat into the perivitelline space of the fertilized oocyte, but sperm could not fuse with the oocyte plasma membrane. The block at the plasma membrane is also found in the nemertean *Cerebratulus* (Kline *et al.*, 1985), in mammals (*e.g.*, Horvath *et al.*, 1993), and in the surf clam *Spisula* (Ziomek and Epel, 1975).

The existence of the electrical block in bivalves was suggested in *Spisula* by Finkel and Wolf (1980), who found that the depolarization of the oocyte plasma membrane occurs soon after insemination and that low-sodium seawater induces polyspermy. A vitelline coat and cortical granules are present in the oocyte cortex in bivalves, but there is no evidence demonstrating the formation of a

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**Abbreviations:** ASW, artificial seawater; DAPI, 4',6-diamidino-2-phenylindole; GalNAc, *N*-acetylgalactosamine; NaFSW, sodium-free artificial seawater; NSW, natural seawater;  $R_{s/o}$ , sperm-oocyte ratio.

fertilization envelope by their structural changes (Longo, 1983; Alliegro and Wright, 1983; Longo *et al.*, 1993); thus the involvement of both structures in the late polyspermy block is uncertain. However, a complete polyspermy block has been demonstrated in oocytes of *Spisula* from which the vitelline coat was removed, suggesting that a complete block to polyspermy occurs at the oocyte plasma membrane in this species (Ziomek and Epel, 1975).

In contrast to *Spisula*, the mussel *Mytilus galloprovincialis* was described as lacking a complete mechanism to block polyspermy (Dufresne-Dubé *et al.*, 1983). Dufresne-Dubé *et al.* (1983) also found induction of polyspermy in *M. galloprovincialis* by lowering the concentration of sodium ions in seawater, but no fertilization potential was observed. We demonstrate here a complete polyspermy block in *Mytilus edulis* and describe its three steps: (1) a fast electrical block, (2) a suppression of the acrosomal reaction, and (3) a block of contact or fusion of the plasma membrane in the gametes.

## Materials and Methods

### Artificial seawater

Artificial seawater (ASW) consisted of 450 mM NaCl, 9.4 mM KCl, 10.2 mM CaCl<sub>2</sub>, 48.2 mM MgSO<sub>4</sub>, 5.4 mM NaHCO<sub>3</sub>. When sodium-free artificial seawater (NaFSW) was prepared, NaCl, KCl, and NaHCO<sub>3</sub> were replaced with 455.4 mM choline chloride (Nacalai Tesque Inc.), 4 mM KCl, and 5.4 mM KHCO<sub>3</sub>, respectively. Low-sodium ASWs were prepared by mixing ASW and NaFSW. The pH was adjusted to 8.3 with 1 N NaOH for ASW or 1 N KOH for NaFSW and low-sodium ASWs just prior to use.

### Gametes

Specimens of the mature mussel *Mytilus edulis* were collected from November to April in the vicinity of Asamushi Marine Biological Station (Aomori Prefecture), Tohoku University, and Misaki Marine Biological Station (Kanagawa Prefecture), University of Tokyo. They were kept in aquaria at 10°C. Spawning of oocytes and sperm was induced by transferring the mussels to warm seawater at 25°C. When the mussels started spawning, they were returned to natural seawater (NSW) at 10°C. The oocytes were washed several times with NSW, ASW, or low-sodium ASWs according to the experiments. Concentrations of oocytes were determined by counting the number of oocytes in 5- $\mu$ l glass capillary tubes. Sperm were collected "dry" and stored at 4°C. Sperm suspensions were prepared by diluting the dry sperm with NSW, ASW, or low-sodium ASWs. Concentrations of sperm in the suspensions were determined by counting the number of sperm, fixed with

1% glutaraldehyde, in a hemacytometer. Sperm-oocyte ratio ( $R_{s/o}$ ) in the medium at insemination was an absolute ratio. All experiments were carried out at room temperature (18°–20°C).

### Assay of polyspermy

To remove supernumerary sperm bound to the surface of the oocyte, the inseminated or re-inseminated oocytes were washed with NSW containing 0.001% sodium dodecyl sulfate at 20 min after the first insemination. Oocytes were subsequently fixed with a 3:1 mixture of methanol and acetic acid for 1 h at room temperature. After the oocytes were washed with distilled water, they were stained with 1  $\mu$ g/ml DAPI (4',6-diamidino-2-phenylindole) (100  $\mu$ g/ml in dimethyl sulfoxide as a stock) for 30 min to observe incorporated sperm nuclei. The rate of polyspermy was indicated by the percentage of oocytes that included multiple sperm nuclei. The mean number of sperm nuclei included in an oocyte was determined by counting the number of decondensed sperm nuclei in an oocyte. At least 100 oocytes were observed under a fluorescence microscope (Olympus, BH-2).

### Re-insemination experiments

When oocytes were inseminated with a low  $R_{s/o}$  (light insemination;  $R_{s/o} = 5 \times 10^2 - 1 \times 10^3$ ), fertilized oocytes were monospermic. At various periods up to 5 min after initial insemination, the monospermic oocytes were inseminated again with a high  $R_{s/o}$  (heavy insemination;  $R_{s/o} = 8 \times 10^3 - 1 \times 10^4$ ) sufficient to give a high rate of polyspermy in initial insemination. The length of time during which the sperm remained monospermic upon heavy re-insemination was taken as the completion time for the polyspermy block.

### Assay of sperm binding

Fertilized and unfertilized oocytes were fixed with 1% glutaraldehyde in NSW at 30 s after the heavy insemination ( $R_{s/o} = 8 \times 10^3 - 1 \times 10^4$ ), and then the number of bound sperm was counted by scanning the entire oocyte surface under a Nomarski microscope (Nikon, OPTIPHOT). Observations were made on at least 100 oocytes per experiment.

### Assay of acrosome reaction

Half of the oocytes from a female were lightly inseminated ( $R_{s/o} = 5 \times 10^2 - 1 \times 10^3$ ). The fertilized oocytes and remaining unfertilized oocytes were washed several times with NSW at 10 min after the initial light insemination. Both unfertilized and fertilized oocytes were inseminated at the  $R_{s/o}$  of  $3.5 - 4.0 \times 10^3$ , and then they

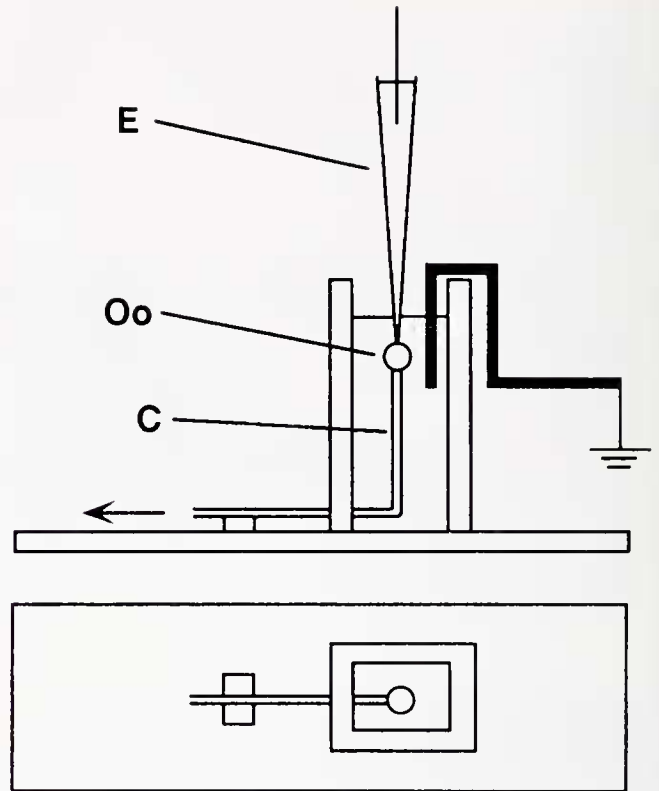
were fixed with 1% glutaraldehyde at 5 min after the insemination. Because the acrosome of *Mytilus* sperm is large (see Fig. 8), acrosome-intact were easily differentiated under a Nomarski microscope from acrosome-reacted sperm and were easily removed after fixation. Thus, an appropriate volume of the suspension was mounted on the glass slide, and the number of acrosome-reacted and acrosome-intact sperm in randomly selected samples of sperm (both bound and unbound on the oocyte surface) was counted under the microscope. As a control, glutaraldehyde-fixed oocytes and sperm were mixed, and the number of acrosome-reacted sperm was counted.

#### Measurement of membrane potential

To make microelectrodes, glass tubing containing a glass fiber was pulled with a microelectrode puller (Narishige, PN-3), and back-filled with 3 M KCl. Resistance of the electrode was 40–60 M $\Omega$ . As shown in Figure 1, the chamber was filled with medium such as NSW, ASW, or low-sodium ASWs, which was connected to ground via an Ag-AgCl electrode. An oocyte was held by sucking on the tip of a capillary that was placed in the chamber. Electrode penetration was achieved by lowering the electrode to the oocyte surface perpendicularly and applying an "oscillating" current. Recordings were made with a microelectrode amplifier (Nihon Kohden, MEZ-7200), an oscilloscope (Hitachi, V-212), and a chart recorder (Hitachi, 200). After measurements of the membrane potential, each oocyte was transferred into a hole of a 96-well culture plate, and the first cleavage (about 80 min after insemination at 18°C) was observed. Oocytes were considered to be polyspermic when the first cleavage was abnormal. When cleavage did not occur, or if the microelectrode resistance changed at the end of a measurement compared to the initial value, the data were not used.

#### Electron microscopy

According to the method of Einsenman and Alfert (1982), gametes were prefixed for 10 min in seawater containing 1% glutaraldehyde (Nacalai Tesque Inc.) and 0.05% osmium tetroxide (TAAB) or in 0.2 M sodium cacodylate buffer (pH 7.2) containing 1% glutaraldehyde and 0.05% osmium tetroxide, 0.1 M NaCl, and 0.35 M sucrose. Then, the samples were fixed for 1 h in 0.2 M sodium cacodylate buffer (pH 7.2) containing 4% glutaraldehyde, 0.1 M NaCl, and 0.35 M sucrose. Postfixation was performed in 0.2 M sodium cacodylate buffer (pH 7.2) containing 1% osmium tetroxide and 0.3 M NaCl for 1 h. After the fixations, the samples were dehydrated in a graded series of ethanol and embedded in Spurr's resin (Polysciences Inc.). Thin sections were cut with a Porter-Blum MT-1 ultramicrotome, stained with 2% aqueous



**Figure 1.** The chamber for the electrophysiological measurements. The oocyte (Oo) was held with a suction capillary tube (C), and the microelectrode (E) was advanced into the oocyte vertically.

uranyl acetate and Reynolds' lead citrate (Reynolds, 1963), and observed with a Hitachi H-500 transmission electron microscope.

## Results

#### Polyspermy block mechanism in the oocyte of *Mytilus edulis*

When oocytes were collected within 30 min after spawning (fresh oocytes) and inseminated with sperm, 90% of the oocytes were monospermic until  $R_{s/o}$  reached  $5 \times 10^3$ ; insemination with higher concentration of sperm ( $R_{s/o}$  is above  $5 \times 10^3$ ) resulted in polyspermy (Fig. 2). The incidence of polyspermy increased when oocytes were aged in seawater before insemination, though the time at which oocytes become polyspermic varied from batch to batch (data not shown). Typical data are shown in Figure 3. When oocytes collected more than 30 min after spawning (old oocytes) were inseminated at a  $R_{s/o}$  of  $2 \times 10^3$ , the number of sperm penetrating the oocyte increased with time, reaching a mean of 3.47 when 60-min-old oocytes were inseminated; insemination with a low  $R_{s/o}$  (9



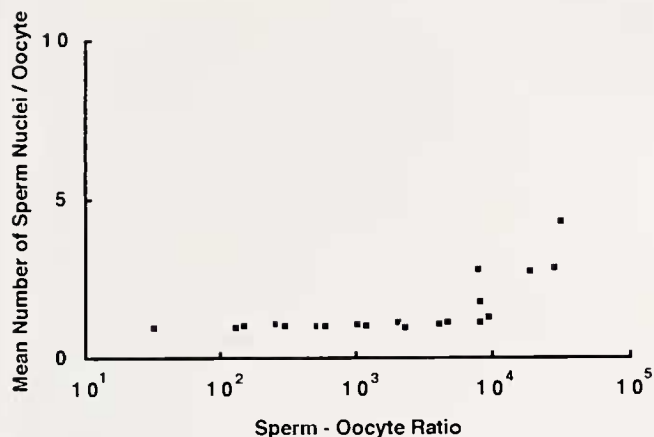


Figure 2. The number of penetrating sperm per fresh oocyte inseminated with various sperm-oocyte ratios. Note that heavy insemination induced polyspermy.

$\times 10^2$ ) resulted in monospermic fertilization even in old oocytes (Fig. 3). The number of penetrated sperm in an oocyte increased when  $R_{s/o}$  was increased and the time of insemination was delayed; the number of penetrated sperm was 2.68, 3.61, and 4.90 when oocytes of the other batch were inseminated at 50, 90, and 120 min after spawning, whereas the number of sperm was 2.46 when fresh oocytes were inseminated with  $R_{s/o}$  of  $1 \times 10^4$  (data not shown). These data suggest that a mechanism to block polyspermy exists in the oocyte of *Mytilus edulis*, and that it weakens with the passage of time after spawning.

The number of penetrated sperm in a fresh oocyte that was inseminated under heavy insemination conditions was 2.79 (Fig. 4). However, when monospermic oocytes made by fertilization with light insemination of fresh oocytes were re-inseminated at a higher  $R_{s/o}$  ( $8 \times 10^3 - 1 \times 10^4$ ; heavy insemination) at 15 and 30 s after the initial light insemination, the number of penetrated sperm per oocyte was 1.38 and 1.18, respectively (Fig. 4). From these results and data from two other batches of oocytes (data not shown), the completion time for polyspermy block was concluded to be 30 s. Thus fresh oocytes acquire a block to polyspermy very rapidly after fertilization.

#### The fast polyspermy block by fertilization potential

Both the depolarization of the oocyte plasma membrane and the polyspermy block in the marine invertebrates and fucoid seaweeds are known to be suppressed in low-sodium ASW (Gould-Somero *et al.*, 1979; Jaffe, 1980; Kline *et al.*, 1985; Brawley, 1991). When the oocytes of *M. edulis* were lightly inseminated in low-sodium ASW, they became polyspermic (Fig. 5). In ASW, 1.04 sperm penetrated the oocyte, but this number was increased by

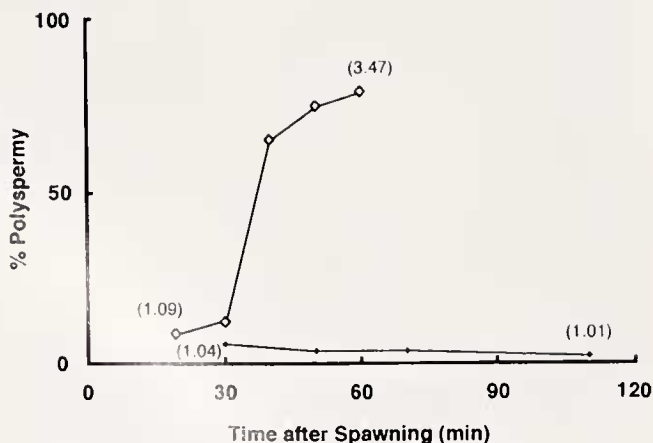


Figure 3. Increase in the rate of polyspermy with passage of time after spawning. The mean number of sperm nuclei per oocyte is in parentheses.  $R_{s/o}$  was  $9 \times 10^2$  ( $\blacklozenge$ ) or  $2 \times 10^3$  ( $\circ$ ).

lowering the sodium ion concentration in ASW, suggesting that a sodium-dependent depolarization causes the fast polyspermy block in *M. edulis*.

It is difficult to insert the microelectrode in *Mytilus* oocytes because the plasma membrane of the oocyte is easily broken by mechanical treatments. In the present study, only eight measurements of the fertilization potential were obtained without damaging the oocytes. The membrane potential of unfertilized *Mytilus* oocytes was  $-66.0 \pm 2.2$  mV ( $n = 5$ ) in NSW (Fig. 6A, Table I). Upon insemination, the plasma membrane of the oocyte rapidly

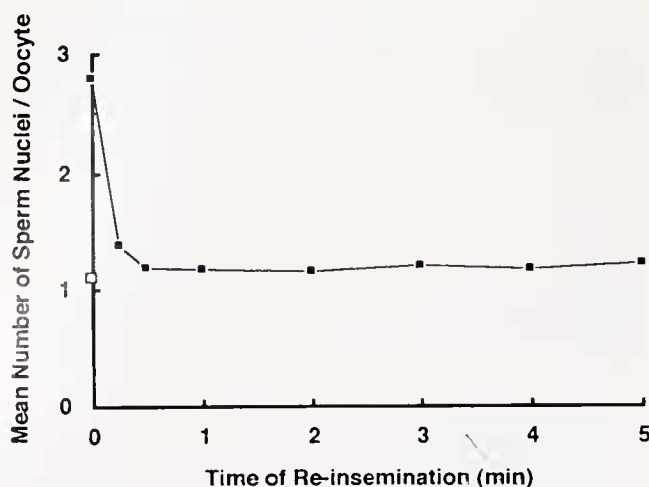


Figure 4. The number of penetrating sperm per oocyte re-inseminated at various times after initial insemination. Values are the mean of 100 oocytes from a single batch. Time zero represents the number of sperm nuclei per oocyte when unfertilized oocytes were heavily ( $\blacksquare$ ) or lightly ( $\square$ ) inseminated.

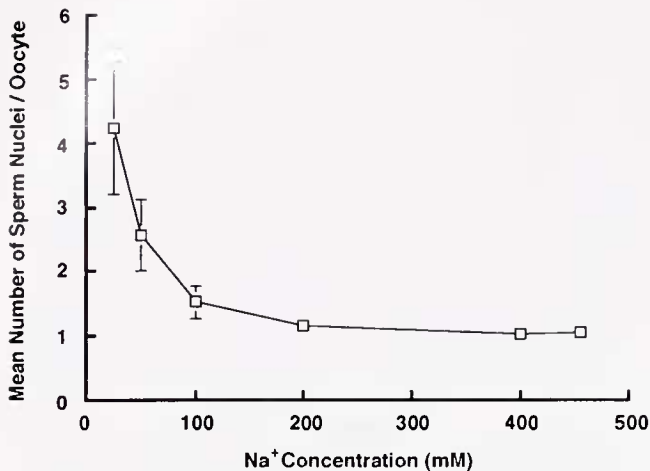


Figure 5. Induction of polyspermy in low-sodium ASW. Oocytes were incubated in low-sodium ASWs for 10 min, and inseminated at  $R_{s/o}$  of  $5 \times 10^2 - 1 \times 10^3$ . Values are the mean  $\pm$  SEM of five experiments.

depolarized to  $+59.4 \pm 2.2$  mV ( $n = 5$ ) and then repolarized to a steady resting potential of  $-68.0 \pm 3.4$  mV ( $n = 5$ ) at  $28.0 \pm 5.2$  s ( $n = 5$ ) after the onset of depolarization (Fig. 6A, Table I). After measurement, all the oocytes became normal 2-cell-stage embryos (Table I). A series of oscillating spikes of depolarization (arrows in Fig. 6A) was observed after the membrane potential returned to a resting potential in all oocytes measured in NSW. These spikes may be due to sperm contacts with the oocyte surface, since the frequency of the spikes increased as the concentration of sperm was increased (data not shown).

When the concentration of sodium in ASW was reduced to 50 mM, the resting potential of unfertilized oocytes was  $-79.3 \pm 5.3$  mV ( $n = 3$ ). The membrane of the oocytes depolarized upon insemination, but the peak value in low-sodium ASW was remarkably lower ( $-56.0 \pm 5.0$  mV,  $n = 3$ ) than that in NSW (Fig. 6B, Table I). All the oocytes transferred into normal ASW after the measurement in low-sodium ASW exhibited abnormal cleavage (Table I). Although we attempted to examine fertilization under current-clamp or voltage-clamp conditions, the experiments were unsuccessful because of the difficulty of electrode penetration.

#### The late polyspermy block

When the fertilized oocytes were heavily re-inseminated ( $R_{s/o} = 8 \times 10^3 - 1 \times 10^4$ ) at 1 min after an initial light insemination ( $R_{s/o} = 5 \times 10^2 - 1 \times 10^3$ ), the number of bound sperm was smaller (0.46) than that in the oocytes initially inseminated at the same  $R_{s/o}$  ( $R_{s/o} = 8 \times 10^3 - 1 \times 10^4$ ) (1.0 at time zero in Fig. 7). The number of bound

sperm was reduced to 0.32, 0.31, or 0.32 when the oocytes fertilized with a light insemination were heavily re-inseminated at 2, 5, or 10 min after initial insemination in NSW, respectively. When fixative (e.g., glutaraldehyde) was added, almost all the acrosome-intact sperm on the oocyte surface were removed (data not shown). These results suggest that a mechanism to prevent sperm binding through suppression of the acrosomal reaction developed shortly after fertilization.

For investigating the relationship between sperm binding and the acrosomal reaction, the rate of the acrosomal reaction of the re-inseminated sperm was investigated (Fig. 8). When unfertilized oocytes were inseminated at a  $R_{s/o}$  of  $3.5 - 4 \times 10^3$  and fixed at 5 min after the insemination, the rate of the acrosomal reaction was 77%. However, this rate was reduced to about 27% when fertilized oocytes (10 min after initial insemination) were re-inseminated at  $R_{s/o}$  of  $3.5 - 4 \times 10^3$  and fixed at 5 min after the re-insemination. Light microscopic observations of living samples also showed that the rate of the acrosome reaction was higher for sperm on the surface of an unfertilized oocyte than for those on a fertilized oocyte (Fig. 8B, C). These results suggest that an acrosomal-reaction-inducing activity is lower on the surface of the fertilized oocytes than on unfertilized oocytes. As a result, sperm hardly undergo the acrosome reaction (Fig. 8) and bind on the surface of the fertilized oocyte (Fig. 7).

When the surfaces of the oocytes that were re-inseminated ( $R_{s/o} = 3.5 - 4 \times 10^3$ ) at 5 min after initial light insemination were observed with a transmission electron microscope, bound sperm had undergone the acrosomal reaction, and the acrosomal process reached the oocyte plasma membrane through the vitelline coat. Typical supernumerary sperm are shown in Figure 9. We examined several serial sections of supernumerary sperm, but we did not find fusion between the supernumerary sperm and the fertilized oocyte. Furthermore, the fertilization cone associated with fertilizing sperm was not observed in these sections or in other single sections. Therefore, we are certain that fertilization of the supernumerary sperm that underwent the acrosomal reaction was prevented at the level of the oocyte plasma membrane.

#### Discussion

Conflicting results have been reported on the polyspermy block mechanism in bivalves. The oocyte of the surf clam *Spisula* has a complete mechanism to block polyspermy (Ziomek and Epel, 1975; Longo, 1976a). Dufresne-Dubé *et al.* (1983), however, obtained monospermic fertilizations in the mussel *Mytilus galloprovincialis* only when the oocytes were inseminated with a very low concentration of sperm at a range of  $R_{s/o}$  between

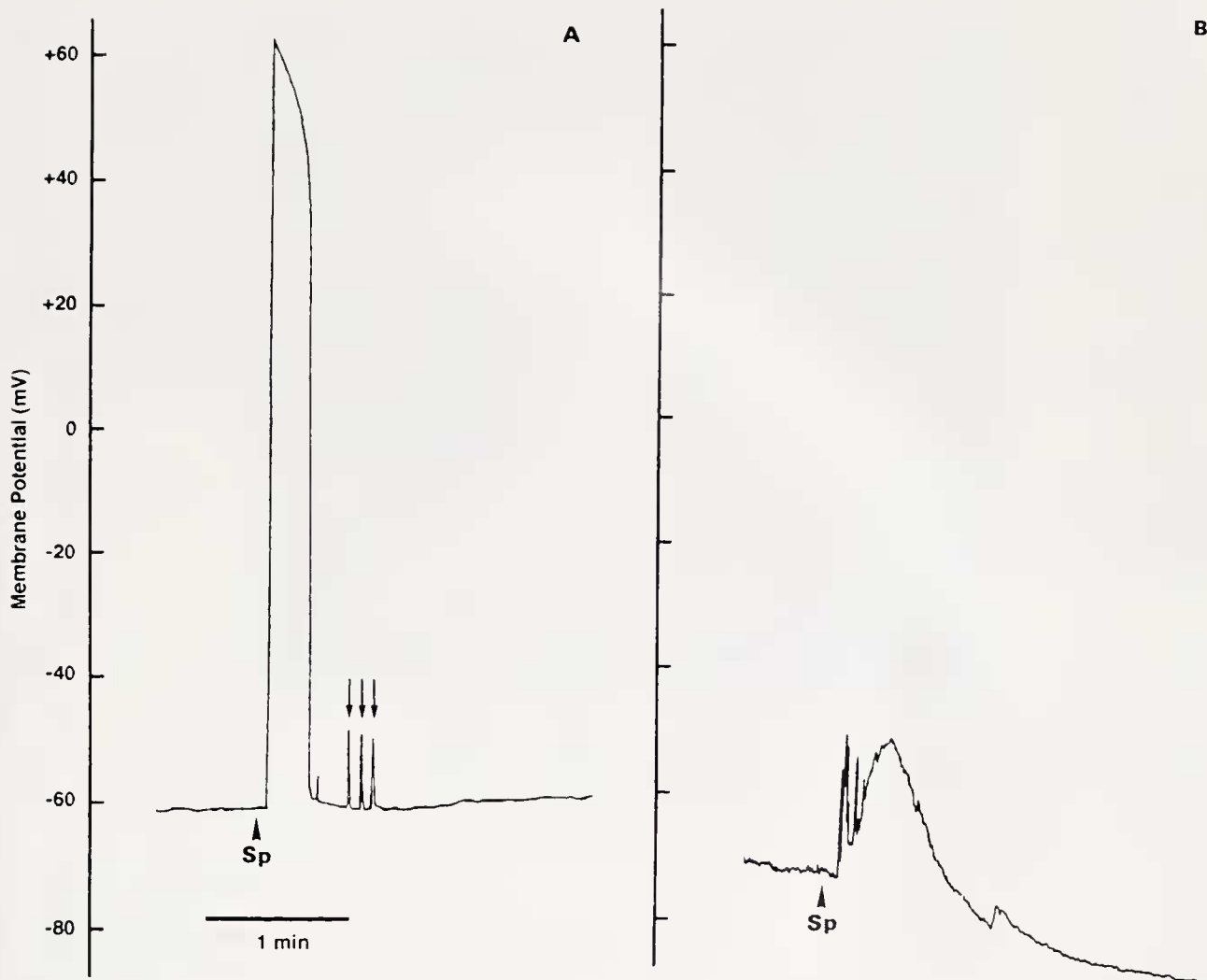


Figure 6. Typical patterns of membrane depolarization of *Mytilus* oocytes at fertilization in NSW (A) or low-sodium ASW (B). Sperm suspension was added to the chamber containing an oocyte at Sp. Sodium concentration in low-sodium ASW was 50 mM. Arrows show depolarization spikes.

Table 1

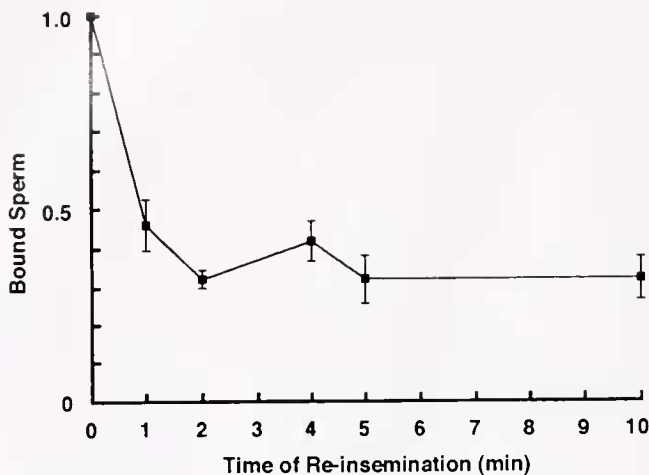
Fertilization potential characteristics in *Mytilus edulis*

Seawater	Resting potential before fertilization (mV)	Resting potential after fertilization (mV)	Peak value (mV)	Duration of fertilization potential <sup>a</sup> (s)	n <sup>b</sup>	% polyspermy
NSW	-66.0 ± 2.2	-68.0 ± 2.2	+59.4 ± 2.2	28.0 ± 5.2	5	0 (0/5)
50 mM-Na <sup>+</sup>	-79.3 ± 5.3	-90.0 ± 2.3	-56.0 ± 5.0	48.7 ± 4.8	3	100 (3/3)

Values are mean ± SE.

<sup>a</sup> Time for which the membrane potential was more positive than resting potential before fertilization.

<sup>b</sup> Number of measurements.



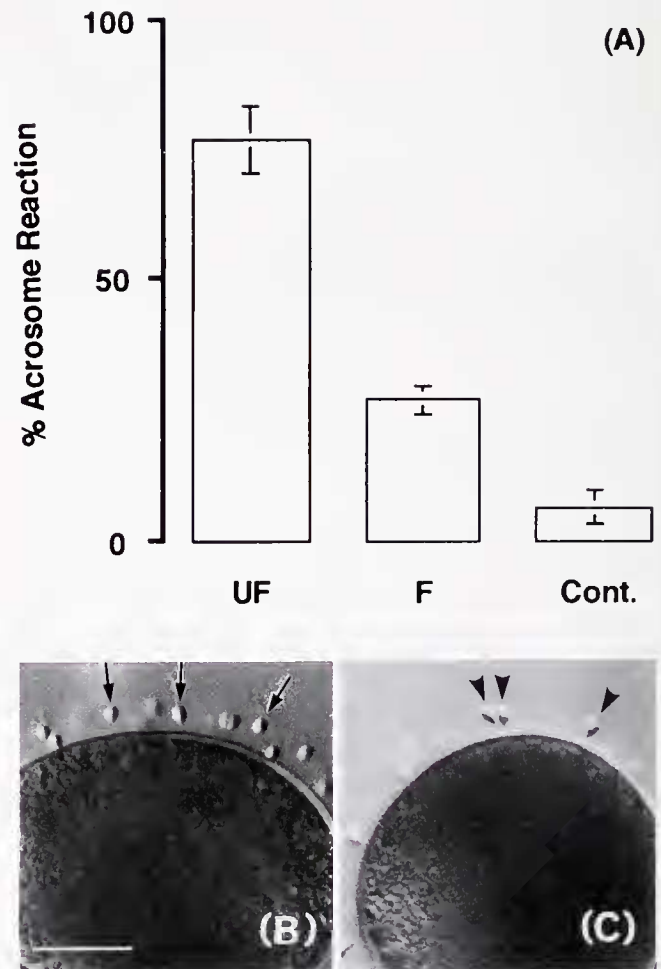
**Figure 7.** Sperm binding to fertilized oocytes. Fertilized oocytes were heavily re-inseminated at appropriate times and fixed at 30 s after re-insemination. Time zero represents heavily inseminated sperm binding to unfertilized oocytes (control). Values are represented as a ratio of the control value (mean  $\pm$  SEM of six experiments).

$10^1$  and  $2 \times 10^2$ , suggesting that no complete block to polyspermy is established in this species. In *Mytilus edulis*, we found here that the oocyte exhibits a complete block to polyspermy after fertilization (Fig. 4), but that the block lasts only 30 min after spawning. Because the incidence of polyspermy increases with time after oocytes are spawned (Fig. 3), use of old oocytes for insemination would lead to the same conclusion for *M. edulis* as Dufresne-Dubé *et al.* (1983) reached for *M. gallo-provincialis*—*i.e.*, that the species lacks a mechanism for complete polyspermy block.

In the brown alga *Fucus* (Brawley, 1991) and in marine invertebrates such as the sea urchin, the starfish, the echiuroid *Urechis*, and the nemertean *Cerebratulus* (Jaffe, 1976, 1980; Miyazaki and Hirai, 1979; Gould-Somero *et al.*, 1979; Kline *et al.*, 1985), the membrane of the oocyte depolarizes at fertilization. In crustaceans, hyperpolarization was observed at fertilization (Goudeau and Goudeau, 1989a, b). All the above reports, except those for crustaceans, also showed that polyspermic fertilization occurs in low-sodium ASW by suppression of the depolarization, and that fertilization is inhibited when the membrane potential is clamped at a positive value. Depolarization of the oocyte plasma membrane also occurred at fertilization in *M. edulis*, and less depolarization and higher polyspermic fertilization were found in low-sodium ASW (Figs. 5 and 6, Table 1), suggesting that an electrical event at the plasma membrane acts as the fast polyspermy block in the oocyte of *Mytilus*.

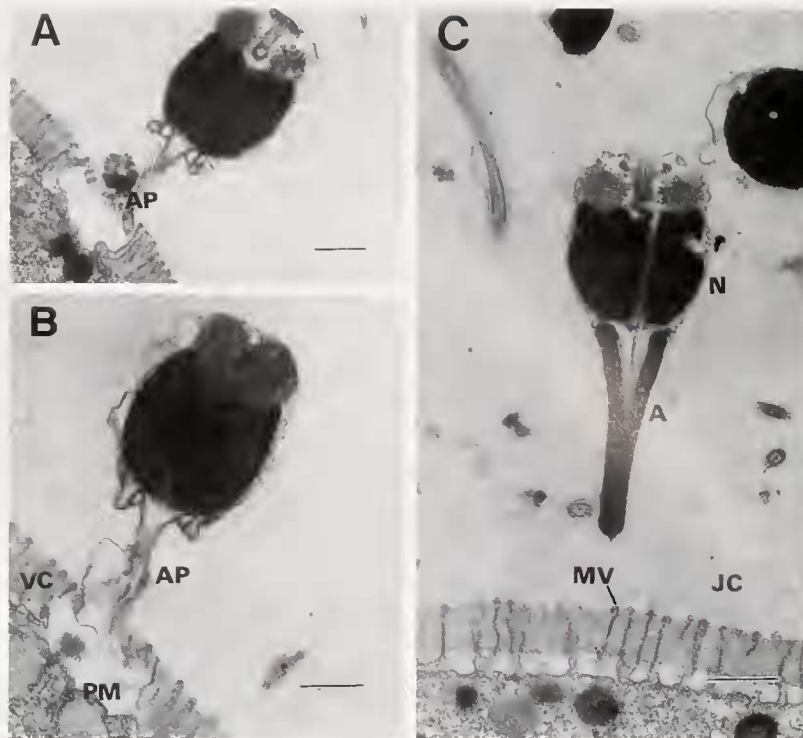
The fast electrical block is not absolute, and thus high sperm concentrations can sometimes overcome it (see

Jaffe and Gould, 1985). An effective late block appears following the fast electrical block (see Fig. 12 in Brawley, 1991). In many species except bivalves, this late block usually accompanies morphological changes in the cortex of the oocyte such as cortical granule breakdown or elevation of the fertilization envelope (Longo, 1983; Jaffe and Gould, 1985). During the formation of the fertilization envelope, sperm-oocyte binding is impaired by enzymes released from the fertilized oocyte, and late polyspermy block is established. For example, during cortical reaction after fertilization, the egg of the sea urchin releases proteases that cause separation of sperm and egg (Vacquier *et al.*, 1972, 1973). Sperm detachment also occurs before



**Figure 8.** Acrosomal reaction in sperm used to inseminate unfertilized or fertilized oocytes. (A) Control represents the acrosome reaction of fixed sperm inseminating fixed oocytes. Values are the mean  $\pm$  SEM of four experiments. (B) On the surface of an unfertilized oocyte, many round acrosome-reacted sperm (arrows) are seen. (C) On the surface of a fertilized oocyte, pear-shaped acrosome-intact sperm (arrowheads) are seen. The photographs were taken within 1 min after re-insemination. When fixative was added, almost all acrosome-intact sperm on the oocyte surface were removed (not shown). Bar = 20  $\mu$ m.





**Figure 9.** Transmission electron micrographs of supernumerary sperm on the surface of the fertilized oocyte. (A, B) Acrosome-reacted sperm at the oocyte surface. Sperm dissolved the vitelline coat (VC), and protruded the acrosomal process (AP) to the oocyte plasma membrane (PM), but the acrosomal process did not fuse with the oocyte plasma membrane. (C) Some acrosome-intact sperm were observed on the surface of the fertilized oocyte without being removed during fixation. A = acrosome. N = nucleus. JC = jelly coat, MV = microvilli. Bar = 1  $\mu$ m.

cell-wall formation, and destruction of the sperm "receptor" with the glycosidase released from the egg after fertilization is hypothesized to occur in *Fucus* (Brawley, 1991). In ascidians (*Ascidia*, *Phallusia*, *Halocynthia*), sperm recognize *N*-acetylglucosamine residues coming out onto the vitelline coat during fertilization (Lambert, 1986; Honegger, 1982, 1986; Matsuura *et al.*, 1993). Although the type of cortical reaction observed in sea urchins is lacking in ascidian eggs (Rosati *et al.*, 1977), the oocytes rapidly release *N*-acetylglucosaminidase after fertilization, blocking the binding of the sperm to the vitelline coat (Lambert, 1986, 1989; Lambert and Goode, 1992).

Neither ascidians (Rosati *et al.*, 1977) nor many bivalve species, including *M. edulis* (Longo, 1983; Alliegro and Wright, 1983; Longo *et al.*, 1993), display the morphological changes of the oocyte cortex seen during fertilization in sea urchins. Nevertheless, in *M. edulis*, the late polyspermy block that follows the fast electrical mechanism is established by 30 s after fertilization since the duration of the fertilization potential is 30 s (Table 1). One stage of the late polyspermy block in the species seems to be inhibition of sperm-oocyte binding through suppression of the ac-

rosomal reaction (Figs. 7 and 8). The acrosome in sperm on the surface of the oocyte can be in one of two states: unreacted or reacted (Figs. 8 and 9). Therefore, it is possible that the oocyte loses its ability to induce an acrosome reaction after penetration by a fertilizing spermatozoon. In *M. galloprovincialis*, the acrosomal reaction is reportedly triggered when sperm recognize *N*-acetylgalactosamine (GalNAc) residues on the oocyte (Focarelli *et al.*, 1991). Perhaps *Mytilus*, like the ascidians (Lambert and Goode, 1992), release some enzyme from its oocyte so that the "receptor" molecule necessary to induce the acrosomal reaction is destroyed or covered, resulting in an inhibition of sperm-oocyte binding through suppression of the acrosomal reaction. In our preliminary study in *M. edulis*, contrary to the report of Focarelli *et al.* (1991), neither fertilization nor sperm-binding were inhibited in the presence of GalNAc (Togo and Morisawa, unpub. data). Treatment of oocytes with GalNAc-binding lectins (DBA and SBA) also failed to inhibit these processes (Togo and Morisawa, unpub. data). Further studies on the "receptor" prerequisite for the induction of the acrosomal reaction in *Mytilus* will be needed.



Some sperm could undergo an acrosomal reaction and bind to the fertilized oocyte (Fig. 7), although the rate of reaction was reduced in re-inseminated sperm (Fig. 8). Electron microscopic observations showed the acrosomal process of sperm penetrating the oocyte by digesting the vitelline coat with sperm lysin (Fig. 9). However, we could observe neither fusion between the acrosomal process and the oocyte plasma membrane nor formation of the fertilization cone associated with fertilizing sperm (Fig. 9), suggesting that the plasma membrane of the oocyte may change after fertilization. This kind of change would block the sperm-oocyte fusion and has been described in other animals such as the echiuroid *Urechis* (Paul and Gould-Somero, 1976), the nemertean *Cerebratulus* (Kline et al., 1985), mammals (e.g., Horvath et al., 1993), and the surf clam *Spisula* (Ziomek and Epel, 1975; Longo, 1976a). Complete polyspermy block at the plasma membrane was reported in *Spisula*: oocytes from which the vitelline coat had been removed were still monospermic (Ziomek and Epel, 1975). In *M. edulis*, however, polyspermic fertilization occurred even when the vitelline coat was removed by actinase E, suggesting that the oocyte of the species has an incomplete mechanism to block polyspermy at the level of the oocyte plasma membrane (Kyojuka and Osanai, 1994). Changes of the plasma membrane at fertilization was reported by Longo (1976b), who observed a twofold increase in the number of particles on the protoplasmic face of the plasma membrane in freeze-fracture replicas of *Spisula* oocytes. Nevertheless, the relationship between the increase in particles and the block to polyspermy at the oocyte plasma membrane remains obscure.

Bivalves such as *Spisula* (Finkel and Wolf, 1980) and *M. galloprovincialis* (Dufresne-Dubé et al., 1983) are thought to produce a fast polyspermy block by depolarizing the oocyte plasma membrane: the same mechanism was found here in *M. edulis*. Data from re-insemination experiments (Ziomek and Epel, 1975; Longo, 1976a) indicate that a late polyspermy block mechanism is found in *Spisula* as well. As reported here, *M. edulis* clearly shows a complete late block following the electrically mediated fast block. Monospermy is ensured after the fast polyspermy block by the collaboration of the two strategies—suppression of the acrosome reaction of the supernumerary sperm and blockage of sperm entry at the plasma membrane.

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